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## Short communication

## Identification of reaction products between drug substances and excipients by HPLC–SPE–NMR: Ester and amide formation between citric acid and 5-aminosalicylic acid

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## ABSTRACT

The reaction between the high-dose drug substance 5-aminosalicylic acid (5-ASA) and the excipient citric acid during storage of an experimental enema preparation has been studied and three isobaric reaction products, i.e., an ester and an amide with non-symmetrically substituted citric acid moieties and a symmetrical amide, were identified by combined use of HPLC–SPE–NMR and HPLC–MS. After storage for 1 week at 70 °C, approximately 5% of the 5-ASA present in the formulation was transformed into these impurities. Storage of the enema for 32 months at room temperature led to loss of approximately 10% of the original amount of 5-ASA, with the ester as the main reaction product.

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## 1. Introduction

The development of methods based on hyphenation of separation techniques and nuclear magnetic resonance (NMR) have greatly improved possibilities of identification of mixture components without the need of prior isolation and purification of individual compounds [1]. Thus, in contrast to well-established HPLC–MS methods, which are characterised by speed and high sensitivity but also by limited information content with respect to details of molecular structure, HPLC–NMR allows unambiguous on-line identification of even very complex structures. A recent extension of the original HPLC–NMR repertoire of experiments (comprising on-flow, stopped flow and loop-storage techniques [1,2]) is HPLC–SPE–NMR, where the analytes are separated from the HPLC mobile phase by means of post-column solid-phase extraction and then submitted, in an automated fashion, to NMR measurements in a deuterated solvent. Combined advantages of analyte focusing, accumulation by multiple SPE collections and the use of deuterated solvents makes HPLC–SPE–NMR capable of providing NMR data of higher quality than what is usually achievable by direct HPLC–NMR methods, i.e., methods where NMR

data are obtained with analytes dissolved in the HPLC mobile phase [2–4]. However, the HPLC–SPE–NMR technique is inherently dependent on the effectiveness of the post-column SPE process [5]. The HPLC–SPE–NMR technique is becoming increasingly popular, especially in natural products research [6–10], food and food supplement analysis [11,12], and pharmaceutical analysis [13], including analysis of drug degradation [14] and drug metabolism [15,16]. In this work, we describe the first example of application of HPLC–SPE–NMR to analysis of reaction products formed by interactions between a drug substance and an excipient upon storage of a pharmaceutical formulation.

For more than half a century, 5-aminosalicylic acid (5-ASA) has been used in the treatment of chronic inflammatory bowel diseases, originally in the form of the prodrug sulphasalazine [17] and subsequently as 5-ASA itself, and in the beginning of the 1980's the development of 5-ASA drugs accelerated [18–20]. Enemas are among the popular formulation forms for colonal delivery and are still very important in the treatment of distal colitis and proctitis [21,22]. The present text deals with the formation of impurities in an enema formulation of 5-ASA, that has been under development by a pharmaceutical company. It was important to elucidate the structures of the impurities in order to eliminate them from the final formulation. 5-ASA is used in relatively large doses and therefore has a potential of formation of relatively high amounts of potentially harmful impurities.

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Because 5-ASA is prone to oxidation, especially in alkaline solution, a number of excipients are usually added to 5-ASA solutions, including ethylenediaminetetraacetic acid, sulfite, and citric acid. The latter is widely used in pharmaceutical formulations as an acidifier, complexing agent or buffer substance. However, citric acid and other di- and polycarboxylic acids can form cyclic anhydrides, which can significantly increase their reactivity in the liquid as well as the solid state [23–25]. Recently, a reaction between 5-ASA and citric acid in a formulation produced by hot-melt technology has been reported, but no final conclusion about the structure of the reaction product was reached [26].

In high dose-drugs, an impurity present in an amount of 0.1% or more relative to the active substance has to be conclusively identified. This identification threshold often poses a considerable analytical challenge. Here, we describe identification of reaction products formed in liquid formulations from 5-ASA and citric acid using HPLC–SPE–NMR supported by HPLC–MS. The study thus addresses the problem of distinction between isomeric products formed with this widely used pharmaceutical excipient and is of potential interest for product control of liquid pharmaceutical formulations of drugs containing hydroxy and amino groups.

## 2. Experimental

### 2.1. Materials

Citric acid monohydrate and formic acid were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Methanol and phosphoric acid were purchased from VWR (Leicester, England). 5-Aminosalicylic acid (5-ASA) and *N*-acetyl-5-aminosalicylic acid

were obtained from Ferring Pharmaceuticals (Copenhagen, Denmark).

### 2.2. High-performance liquid chromatography–mass spectrometry (HPLC–MS)

HPLC–MS experiments were performed on an Agilent 1100 HPLC/MSD system (Agilent Technologies, Palo Alto, CA, USA). The chromatographic system consisted of a Phenomenex Luna  $2 \times 100$  mm  $3 \mu\text{m}$   $\text{C}_{18}$  column kept at  $40^\circ\text{C}$  and the mobile phases A (10% MeOH in water, added 0.1%  $\text{HCOOH}$ ) and B (90% MeOH in water, added 0.1%  $\text{HCOOH}$ ). The gradient elution profile was 0% B at 0 min, 20% B at 10 min, 50% B at 20 to 24 min, 0% B at 24.1 min, with a flow-rate of 0.3 mL/min and a total runtime of 42 min. UV traces were recorded at 240 nm. The MSD was used in positive electrospray mode, drying gas flow 12 L/min, nebuliser pressure 40 psi, drying gas temperature  $350^\circ\text{C}$ , capillary voltage 4 kV and fragmentor 80 V. The MSD was programmed to scan from 100–1200 a.m.u. with a cycle time of 1.08 s. Samples were diluted appropriately with water; the total concentration was typically 0.5 mg/mL and the injection volume was 5  $\mu\text{L}$ .

### 2.3. High-performance liquid chromatography–solid-phase extraction–nuclear magnetic resonance (HPLC–SPE–NMR)

The HPLC–SPE–NMR system consisted of an Agilent 1100 series chromatograph (quaternary pump, autosampler, column oven, photodiode array detector), a Knauer K100 Wellchrom post-column solvent delivery pump (Berlin, Germany), a Spark Prospekt 2 device (Emmen, Holland), and a Bruker Avance 600 MHz NMR spectrom-

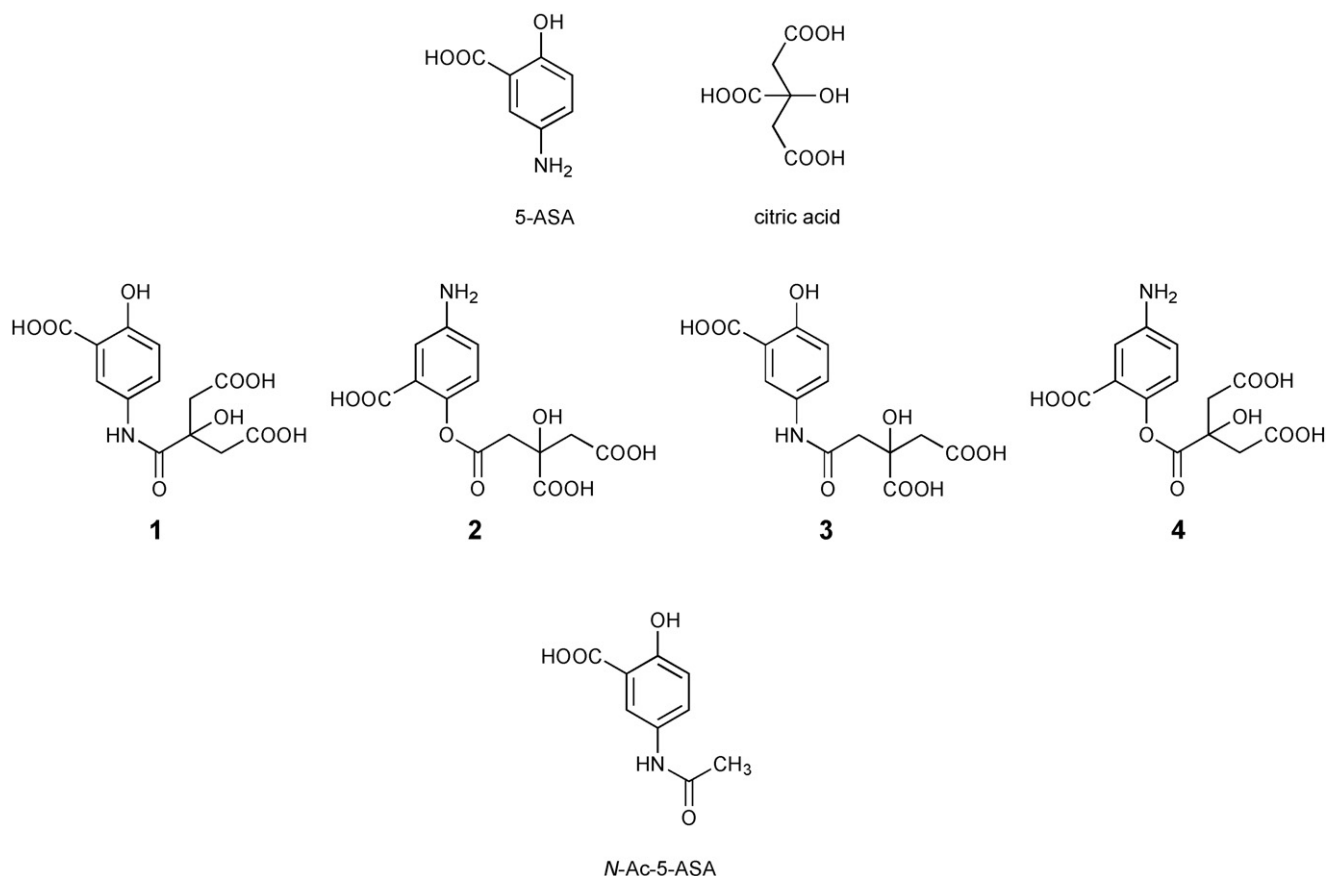
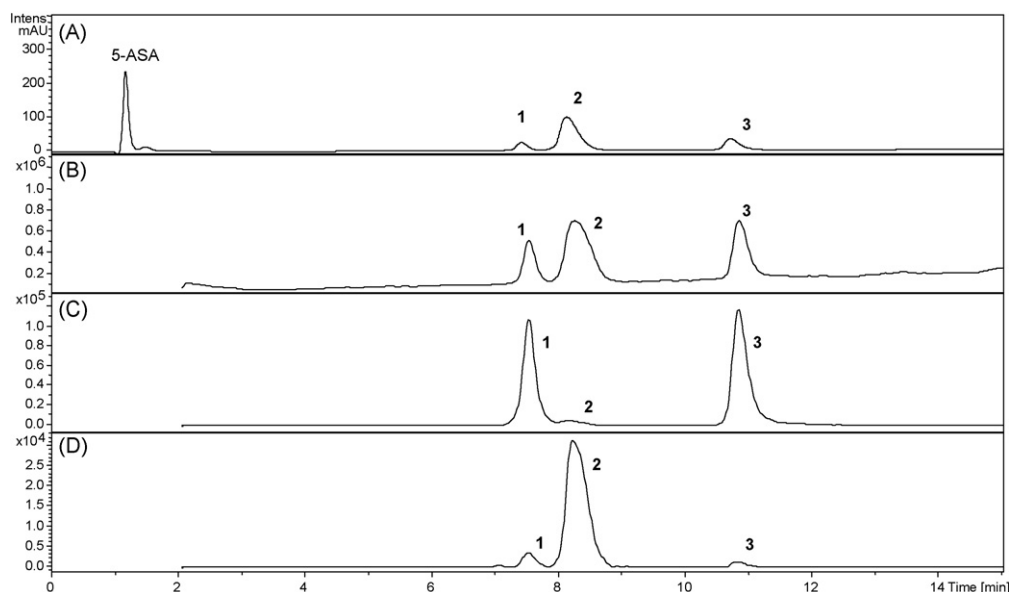


Fig. 1. Structures of 5-ASA, citric acid, their esters and amides (1–4), and the model compound *N*-acetyl-5-ASA.



**Fig. 2.** HPLC–UV–MS chromatograms of enema formulation containing 5-ASA and citric acid after storage for 32 months at ambient temperature. (A) UV trace at 240 nm. (B) Total ion chromatogram. (C) Extracted ion chromatogram  $m/z$  328. (D) Extracted ion chromatogram  $m/z$  310. Chromatographic conditions: Phenomenex Luna C<sub>18</sub> column eluted with a gradient of methanol in water (both containing HCOOH) with UV and positive-ion electrospray MS detection (for details, see Section 2.2).

eter (Rheinstetten, Germany) equipped with a 3 mm (30  $\mu$ L active volume)  $^1\text{H}\{^{13}\text{C}\}$  flow probe. The separations were performed at 40 °C on a 10  $\times$  150 mm Phenomenex Luna 3  $\mu$ m C<sub>18</sub> column with a flow-rate of 0.8 mL/min. The mobile phase consisted of 35% MeOH in water with 0.1% HCOOH added. The post-column diluent was 0.1% HCOOH in water, flow rate 2 mL/min. Injection volume was 100  $\mu$ L. Threshold absorbance levels for analyte trapping were defined at 240 nm and six cumulative trappings on 2  $\times$  10 mm HySphere C<sub>18</sub> HD cartridges were performed. Compounds were eluted from the SPE cartridges with methanol- $d_4$ . The  $^1\text{H}$  NMR spectra were calibrated to the residual solvent signal of methanol- $d_4$  at  $\delta$  3.31.

#### 2.4. Stability of 5-ASA formulations

An enema formulation containing 5.00 g 5-ASA, 0.10 g sodium edetate, 1.00 g sodium pyrosulfite, 5.00 g citric acid monohydrate, 1.75 g sodium hydroxide and 490 g purified water was investigated. The pH of the suspension was 4.8. The formulation was stored for 1 week at 70 °C. Another sample was stored at ambient temperature for 32 months. In both cases, the suspension was diluted ten-fold with water before being injected into the HPLC–MS system. To assess stability of the reaction products towards base, 500  $\mu$ L of the aged preparation was mixed with 500  $\mu$ L of 0.500 M NaOH; after 5 min at ambient temperature an aliquot was mixed with equal amount of 0.500 M phosphoric acid, diluted four-fold with water, and analysed immediately by HPLC–MS. A model mixture, containing 100 mg 5-ASA and 100 mg citric acid monohydrate in 1 mL

water, was heated for 3 h at 70 °C and the supernatant analysed by HPLC–SPE–NMR.

### 3. Results and discussion

In a pharmaceutical formulation containing both 5-ASA and citric acid, at least four primary reaction products **1–4**, i.e., two amides (**1** and **3**), and two phenol esters (**2** and **4**), can be expected to be formed (Fig. 1). Formation of an ester of 5-ASA with the hydroxy group of citric acid is not expected, as tertiary alcohols do not form esters by direct esterification.

During a stability study of an enema formulation containing 5-ASA as well as citric acid, formation of three new peaks (Fig. 2) was observed by HPLC–MS (retention times 7.6, 8.3 and 10.9 min), and the same three products were formed upon treatment of the enema at 70 °C for a week. All the peaks had  $m/z$  328, corresponding to reaction products between 5-ASA and citric acid, although a water loss ( $m/z$  310) was so pronounced for the peak with retention time 8.3 min that intensity of the quasimolecular ion was low (Fig. 2).

In order to elucidate structures of the reaction products, a mixture of 5-ASA and citric acid was heated for 3 h at 70 °C and the mixture was investigated by HPLC–SPE–NMR. An HPLC–MS investigation revealed reaction products with retention times and mass spectra identical to those formed in the real enema upon storage at room temperature or subjected to accelerated decomposition at 70 °C.  $^1\text{H}$  NMR data for all three products obtained from spectra recorded in the HPLC–SPE–NMR mode are collected in Table 1. The spectra are shown in Fig. 3.

**Table 1**

$^1\text{H}$  NMR spectroscopic data ( $\delta$  values with intensities, multiplicities and coupling constants in Hz in parentheses) for main reaction products between 5-ASA and citric acid, and for authentic *N*-acetyl-5-aminosalicylic acid (methanol- $d_4$ ).

Compound	H-3	H-4	H-6	Citric acid CH <sub>2</sub>	<i>N</i> -acetyl CH <sub>3</sub>
<b>1</b>	6.90 (1H; d; 8.8)	7.62 (1H; dd; 8.8, 1.9)	8.09 (1H; d; 1.9)	2.77 (2H; d; 15.6) 2.98 (2H; d; 15.6)	–
<b>2</b>	7.05 (1H; d; 8.8)	7.40 (1H; dd; 8.8, 2.4)	7.80 (1H; d; 2.4)	2.98 (1H; d; 16.9) 3.13 (1H; d; 16.9)	2.84 (1H; d; 18.4) 3.17 (1H; d; 18.4)
<b>3</b>	6.88 (1H; d; 8.9)	7.60 (1H; dd; 8.9, 2.1)	8.05 (1H; d; 2.1)	2.83 (1H; d; overlap) 2.97 (1H; d; 16.9)	2.83 (1H; d; overlap) 2.90 (1H; d; 14.2)
<i>N</i> -Ac-5-ASA	6.88 (1H; dd; 8.9, 0.3)	7.59 (1H; dd; 8.9, 2.7)	8.06 (1H; dd; 2.7, 0.3)	–	2.10 (3H; s)

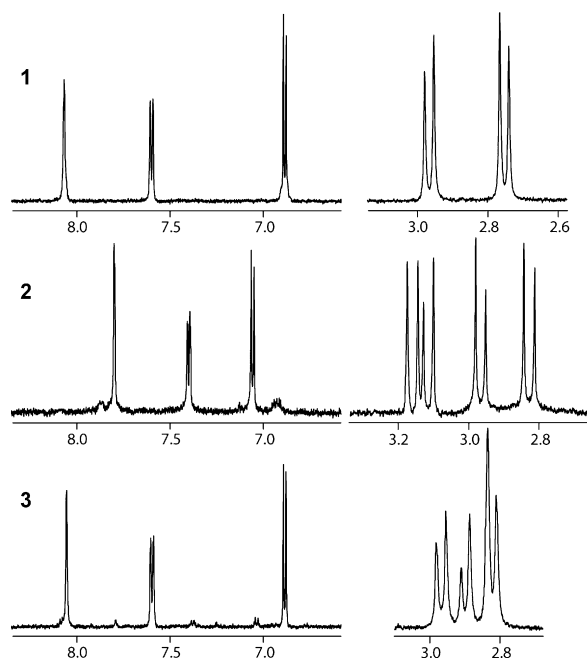


Fig. 3.  $^1\text{H}$  NMR signals of reaction products between 5-ASA and citric acid obtained in HPLC–SPE–NMR mode (600 MHz, methanol- $d_4$ , six trappings on  $\text{C}_{18}$  HD SPE cartridges).

It is apparent that the  $^1\text{H}$  NMR spectrum recorded with the first of the three impurity peaks shows the presence of a symmetrically substituted citric acid moiety, as the spectrum contains one AB spin–spin coupling system with a geminal coupling constant between the diastereotopic methylene protons. By contrast, the spectra of the remaining compounds show the presence of two AB systems each. The first impurity peak can therefore be either the amide **1** or the ester **4**. The distinction between the ester and the amide can be made on the basis of chemical shifts within the benzene ring. Thus, comparison of the  $^1\text{H}$  NMR spectrum with that of authentic *N*-acetyl-5-aminosalicylic acid (600 MHz spectrum in methanol- $d_4$  recorded using a 5 mm tube) demonstrated, that the chemical shifts of the benzene ring protons of the two compounds are almost identical (Table 1). Therefore, the reaction product with the shortest retention time is the symmetric citric acid amide (**1**).

The remaining two impurity peaks must thus correspond to compounds **2** and **3** having non-symmetrically substituted citric acid moiety. Again, comparison of their  $^1\text{H}$  NMR spectra with that of *N*-acetyl-5-aminosalicylic acid (Table 1) demonstrated that the major impurity is the ester **2** and the impurity with the longest retention time is the amide **3** (cf. Fig. 2). Additional evidence was obtained by taking advantage of the fact, that phenol esters in contrast to amides are very labile towards alkaline hydrolysis. Thus, brief incubation of the aged enema formulation with alkali followed by acidification led to the disappearance of the major impurity peak corresponding to compound **2**, whereas the two minor peaks corresponding to compounds **1** and **3** were unchanged.

In the aged enema formulation (32 months), the amount of 5-ASA converted to **1**, **2** and **3** was approximately 1.1%, 6.6% and 1.7%, respectively (estimated by HPLC assuming the same absorptivity of the products at 240 nm). Thus, almost 10% of the original amount of 5-ASA was lost via the reaction with citric acid. In the accelerated experiment (1 week at 70 °C), the approximate amount of impurities was 5%, distributed as 0.5%, 4.0%, and 0.5% of **1**, **2** and **3**, respectively.

## 4. Conclusions

The impurity profile for an enema formulation of 5-ASA with citric acid was investigated and the impurities formed upon storage were identified as compounds **1**, **2** and **3**. Formation of the symmetrical ester **4** was not observed, presumably because of steric hindrance around the central carboxy group of citric acid and thus negligible equilibrium concentration of this phenol ester. The identity of the products was established using the HPLC–SPE–NMR technique. Use of this hyphenated technique allowed acquisition of  $^1\text{H}$  NMR data with a model formulation subjected to accelerated decomposition, leading to identification of the isobaric reaction products.

Because of a relatively rapid and extensive formation of reaction products between 5-ASA and citric acid, the use of citric acid in liquid 5-ASA formulations is not recommended. Thus, based on these findings, further development of a formulation of 5-ASA containing citric acid was abandoned and replaced with another formulation.

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